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Activity of Cefepime against Ceftazidime- and Cefotaxime-Resistant Gram-Negative Bacteria and Its Relationship to β-Lactamase Levels

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One hundred clinical isolates resistant to ceftazidime and/or cefotaxime were examined for susceptibility to cefepime. The most frequently encountered ceftazidime-cefotaxime-resistant strains belonged to the genera *Enterobacter*, *Pseudomonas*, and *Citrobacter*. Among these strains, 92% were resistant to cefoperazone, 91% were resistant to cefotaxime, 84% were resistant to ceftazidime, and 6% were resistant to cefepime. Of the members of the family *Enterobacteriaceae*, 57% were resistant to ceftriaxone. The six strains resistant to cefepime were all *Pseudomonas aeruginosa* and were resistant to both cefotaxime and ceftazidime. Cefepime-resistant *P. aeruginosa* strains had exceptionally high levels of β-lactamase activity, higher than the levels found in strains resistant to ceftazidime but susceptible to cefepime. The β-lactamases from the cefepime-resistant strains were type I (Richmond-Sykes), were constitutively produced, and did not have increased affinity or hydrolytic activity for cefepime. Thus, cefepime was active against most gram-negative bacteria which have developed resistance to the broad-spectrum cephalosporins, and resistance to cefepime in *P. aeruginosa* appears to be associated with higher β-lactamase levels than in cefepime-susceptible strains.

Resistance to broad-spectrum cephalosporins was reported early during their clinical use; e.g., strains of *Enterobacter*, *Serratia*, and *Pseudomonas* species that are resistant to ceftazidime and cefotaxime have emerged during therapy with these antibiotics (1, 7, 13). These strains were resistant to multiple β-lactam antibiotics, including all of the broad-spectrum cephalosporins. In general, these organisms possess chromosomally mediated β-lactamases (16).

Although it was initially perceived that the broad-spectrum cephalosporins were β-lactamase stable, hydrolysis of these drugs does occur at very low rates at physiologically relevant concentrations (20). It has been suggested that in strains with derepressed synthesis of the enzyme, the β-lactamase levels may be sufficient to result in resistance when drug inactivation by hydrolysis exceeds the rate of drug penetration from the outside (10, 20). Although this may be the primary reason for resistance to most broad-spectrum cephalosporins, the results of experiments with ceftazidime (20) were less conclusive (12). Nonhydrolytic interference may be an additional factor in resistance to ceftazidime, which has high affinity to chromosomally encoded β-lactamases and is hydrolyzed at very low rates by those enzymes (12, 19).

A newer cephalosporin that is under development, cefepime (BMY 28142), which has a broad spectrum of activity against gram-negative and gram-positive bacteria (6), has poor affinities for β-lactamases and high resistance to enzymatic hydrolysis (5, 12). In this study, we examined the activity of cefepime against strains resistant to cefotaxime, ceftazidime, or both, as well as relevant β-lactamase kinetic parameters and outer membrane permeability.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study were clinical isolates obtained from hospitals throughout the United States during 1987. These isolates were resistant to either cefotaxime (MIC, ≧64 μg/ml) or ceftazidime (MIC, ≧32 μg/ml) or both.

Antibiotics. Cefepime, sulfate salt, was prepared at Bristol-Myers Research Institute, Tokyo, Japan, and Bristol-Myers Co., Syracuse, N.Y. Cefotaxime was provided by Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.; ceftazidime was obtained from Glaxo, Inc., Research Triangle Park, N.C.; cefoperazone was from Pfizer Inc., Groton, Conn.; and ceftriaxone was from Hoffmann-La Roche Inc., Nutley, N.J.

Growth-inhibitory activity. MICs were determined by an agar dilution method in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards (9). The inoculum contained ca. 5×10^5 CFU per spot (range, 2×10^5 to 8×10^5 CFU per spot), and the MIC was read as the lowest concentration of antibiotic that prevented visible growth or yielded less than six discrete colonies after 18 to 24 h of incubation at 35°C. All studies were performed in triplicate on different days.

β-Lactamase assay. The β-lactamase assay was performed spectrophotometrically (15, 21) by measuring the change in absorbance at the appropriate wavelength for each substrate. The wavelengths and the millimolar absorbancy differences used to calculate the rates of hydrolysis were 260 nm and 10.2 mM/cm for cephaloridine, 264 nm and 7.25 mM/cm for cefotaxime, 257 nm and 13.8 mM/cm for ceftazidime, 260 nm and 8.2 mM/cm for cefepime, and 510 nm and 19 mM/cm for nitrocefin. β-Lactamase activity was measured in 2 ml of a 100 μM substrate in 50 mM phosphate buffer, pH 7.0. One enzyme unit is defined as that amount of enzyme which hydrolyzes 1 μmol of nitrocefin per min at room temperature. The K_i values were calculated by the method of Dixon (11) from the rates of nitrocefin hydrolysis at various concentrations of cefepime and ceftazidime. Nitrocefin and inhibitor were added simultaneously to yield values reflecting initial binding interactions.

Induction of β-lactamase. Overnight cultures were diluted 1:20 into fresh brain heart infusion broth to a final volume of

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TABLE 1. Cefepime susceptibilities of ceftazidime- and/or cefotaxime-resistant clinical isolates

Organism (no. tested)	No. of resistant strains[a]				
	Cefepime	Ceftazidime	Cefotaxime	Cefoperazone	Ceftriaxone
P. aeruginosa (32)	6	17	30	28	NT[b]
E. cloacae (30)	0	30	30	27	27
E. aerogenes (17)	0	16	13	17	2
C. freundii (9)	0	9	7	8	6
Others (12)[c]	0	12	11	11	4
Total/no. tested	6/100	84/100	91/100	91/99	39/68

[a] The MIC breakpoints for resistance were as follows: cefepime, ≧32 μg/ml; ceftazidime, ≧32 μg/ml; cefotaxime, ≧63 μg/ml; cefoperazone, ≥63 μg/ml; ceftriaxone, ≥63 μg/ml.

[b] NT, Not tested.

[c] The 12 strains consisted of 1 *E. coli*, 2 *M. morganii*, 2 *K. pneumoniae*, 4 *S. marcescens*, and 3 *A. calcoaceticus* isolates. One *S. marcescens* isolate was not included for cefoperazone and ceftriaxone because of loss on transfer.

20 ml. After 2 h of incubation on a rotary shaker at 35°C, 10 μg of cefoxitin per ml was added and incubation was continued for another 2 h. Late-exponential-phase cells were harvested by centrifugation (16,300 × *g*, 10 min, 4°C), suspended in 10 ml of 50 mM phosphate buffer (pH 7.0), sedimented again (31,000 × *g*, 10 min, 4°C), and suspended in 3 ml of 50 mM phosphate buffer (pH 7.0). The cells were disrupted by sonication (twice for 50 s each time at 0°C; microtip output, 5; Vibracell 600; Sonics and Materials, Danbury, Conn.). β-Lactamase was measured in the crude extract obtained after removal of cellular debris by centrifugation (31,000 × *g*, 15 min, 4°C).

MICs after imipenem induction (as detailed for cefoxitin induction except at 1/8 of the MIC of imipenem) were determined by microtiter dilution assay (10) directly from induced cell cultures.

Permeability measurement. The relative permeabilities of the bacterial strains were estimated by measuring the rates of hydrolysis of nitrocefin by intact cells and by their sonic extracts (8). Assays with intact cells were performed immediately after cell harvest to minimize enzyme leakage from the bacteria.

Protein determination. Protein concentrations were determined by using the Pierce BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.

Isoelectric focusing. Isoelectric focusing of the supernatants from centrifuged cell sonic extracts was performed on LKB Ampholine polyacrylamide plates (pH 3.5 to 9.5) at 10°C by using an LKB 2117 Multiphor and an LKB 2103 power supply. After pre-equilibration for 15 min at 30 W, 1,800 V, and 50 mA, samples were loaded and run for 1.5 h in the constant-power mode at the same setting. β-Lactamase activity was visualized by soaking the gel in 1 mM nitrocefin in 50 mM KH_2PO_4 (pH 7.0). An isoelectric-focusing calibration kit (pH 5 to 10.5) (Pharmacia Fine Chemicals, Piscataway, N.J.) was used for standards.

RESULTS

Susceptibility of clinical isolates. One hundred ceftazidime- and/or cefotaxime-resistant clinical isolates were evaluated. The most frequently encountered resistant strains belonged to the genera *Enterobacter*, *Pseudomonas*, and *Citrobacter* (Table 1). All 30 *Enterobacter cloacae* strains were resistant to both ceftazidime and cefotaxime, 27 were resistant to ceftriaxone and cefoperazone, and all were susceptible to cefepime (MIC for 90% of the strains tested, 7.4 μg/ml). Of the 17 *Enterobacter aerogenes* strains, all were cefoperazone resistant, 16 were ceftazidime resistant, and 13 were cefotaxime resistant. *E. aerogenes* strains were more susceptible to cefepime, with an MIC for 90% of the strains tested of 1.1 μg/ml. Of the 32 *Pseudomonas aeruginosa* strains, 17 were ceftazidime resistant and most were resistant to cefoperazone and cefotaxime; the remaining strains were moderately susceptible to cefoperazone and cefotaxime. These strains were not tested against ceftriaxone, since ceftriaxone has poor antipseudomonal activity. In contrast, only six *P. aeruginosa* strains had a cefepime MIC of 32 μg/ml or greater; all six were resistant to cefotaxime and ceftazidime. Three of the six strains (A25058, A26316, and A26403) grouped as cefepime resistant (MIC, ≧32 μg/ml) in this study occasionally exhibited cefepime MICs of 16 μg/ml.

The nine *Citrobacter freundii* strains were all resistant to ceftazidime, eight were resistant to cefoperazone, seven were resistant to cefotaxime, six were resistant to ceftriaxone, and none were resistant to cefepime (maximum MIC, 4 μg/ml). Of the 12 ceftazidime-cefotaxime-resistant *Escherichia coli*, *Morganella morganii*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Acinetobacter calcoaceticus* isolates tested, all were susceptible to cefepime.

Of the 100 ceftazidime-cefotaxime-resistant strains tested overall, 91% were cefotaxime and cefoperazone resistant while the other 9% were moderately susceptible to both drugs. Eighty-four percent were resistant to ceftazidime, and only 6% were resistant to cefepime. Interestingly, all six strains resistant to cefepime were *P. aeruginosa*.

Characterization of *P. aeruginosa* clinical strains. The 6 cefepime-resistant *P. aeruginosa* strains were compared with 10 cefepime-susceptible *P. aeruginosa* strains, some of which were additional isolates not included in Table 1. The MICs of cefepime, ceftazidime, and cefotaxime against these 16 strains are listed in Table 2. Four strains (A21213, A9843, A20574, and A21481) were susceptible to all three cephalosporins; strains A20599 and A26382 were cefotaxime resistant but cefepime and ceftazidime susceptible. The remaining cefepime-susceptible strains (A26401, A24291, A26365, and A26144) were resistant to both cefotaxime and ceftazidime.

As a relative measure of β-lactam permeability, nitrocefin hydrolysis by intact cells and their sonic extracts was measured. While there was strain-to-strain variability, there were no readily apparent differences between cefepime-resistant and cefepime-susceptible–ceftazidime-resistant *P. aeruginosa* strains (Table 2). In strains with low levels of β-lactamase, this permeability parameter was indeterminable since there was no measurable hydrolysis of nitrocefin by whole cells.

There were notable differences in the noninduced β-lactamase levels present in strains with different cephalosporin susceptibility patterns (Table 2). In cefotaxime-susceptible strains and strains resistant only to cefotaxime but not to ceftazidime or cefepime, β-lactamase activity was either not detectable or low (7.8 to 13.3 mU/mg). Cefepime-susceptible strains resistant to cefotaxime and ceftazidime exhibited higher β-lactamase levels of 87 to 476 mU/mg. Cefepime-resistant strains had even higher levels of β-lactamase activity of 500 to 4,000 mU/mg. The three cefepime-resistant strains (A25058, A26316, and A26403) with cefepime MICs (16 to 32 μg/ml) near the breakpoint

TABLE 2. MICs and β-lactamase parameters of cefepime-susceptible and cefepime-resistant *P. aeruginosa* clinical isolates

P. aeruginosa strain	MIC (μg/ml)			β-Lactamase sp act (mU/mg)			K_i (μM)		β-Lactamase pI(s)
	Cefepime	Ceftazidime	Cefotaxime	Noninduced[a]	Induced[b]	Sonic extract/intact cells[c]	Cefepime	Ceftazidime	
Cefepime susceptible									
A21213	4	1	2	<2	90	I			9.0
A9843	2	2	16	12.9	108	I			
A20574	2	1	16	<2	67	I			
A21481	2	16	16	13.3	120	I	2,040	NC[d]	8.55, 8.8
A20599	4	2	64	10.3	320	I	990	40	8.5, 8.8
A26382	8	4	128	7.8	3.8	1.1			8.5
A26401	8	32	64	476	370	34.3	2,700	140	8.5
A24291	16	64	>128	87	160	0.4			
A26365	16	64	>128	208	444	I			
A26144	16	32	64	267	324	11.1			
Cefepime resistant									
A25058	32	>128	>128	1,000	2,037	58.1	751	147	8.5
A26316	32	32	>128	514	373	15.6	930	85	8.5, 8.8
A26403	32	64	>128	500	800	3.5	2,054	80	8.8
A26363	32	128	>128	950	1,143	44.8	2,360	48	8.8
A26404	64	>128	>128	1,286	1,467	1.2	1,370	66	8.5
A26145	128	>128	>128	4,000	3,250	10.4	1,480	98	8.5

[a] Measured in supernatant of sonic extract.
[b] Measured in supernatant of sonic extract from cells grown for 2 h in 10 μg of cefoxitin per ml.
[c] Ratio of nitrocefin hydrolyzed by sonic extract to that hydrolyzed by intact cells. I, Indeterminable (no measurable activity with intact cells).
[d] NC, No change (see text).

exhibited lower β-lactamase levels (500 to 1,000 mU/mg) than did the three cefepime-resistant strains (A26363, A26404, and A26145), which consistently had high cefepime MICs (32 to 128 μg/ml). On average, cefepime-resistant *P. aeruginosa* isolates contained at least 5-fold-higher levels of β-lactamase than did cefepime-susceptible–ceftazidime-resistant strains and 150-fold-higher β-lactamase levels than did ceftazidime-susceptible–cefotaxime-resistant isolates.

Significant induction of β-lactamase by cefoxitin occurred only in cefotaxime-susceptible strains and in A20599, which was cefotaxime resistant but ceftazidime and cefepime susceptible. The induced levels of β-lactamase were about 10-fold higher than those observed in the noninduced state in these strains. In the ceftazidime-resistant strains tested, no significant β-lactamase induction was observed, suggesting that the high β-lactamase levels in these strains were constitutively produced. Thus, the cefepime-resistant clinical strains of *P. aeruginosa* differed from cefepime-susceptible strains in that they produced exceptionally high levels of β-lactamase. These cefepime-resistant strains were stably derepressed in their β-lactamase production.

Isoelectric focusing of the supernatants from centrifuged cell sonic extracts of a selected number of strains yielded only a single nitrocefin-positive band with a pI of either 8.5 or 8.8 for most strains (Table 2). In three strains, two nitrocefin-positive bands appeared, although one band always appeared more predominant. The β-lactamases in resistant isolates were identical to those found in cefepime-susceptible isolates but were produced in greater quantities.

In both cefepime-susceptible and -resistant strains, cefepime had a lower affinity for β-lactamase than did ceftazidime (Table 2). The K_is for ceftazidime (40 to 147 μM) were lower than those for cefepime (751 to 2,700 μM), with the exception of the enzyme from strain A21481, for which there was no notable change in nitrocefin hydrolysis with the addition of ceftazidime up to 4 mM. However, the β-lactamase of A21481 may differ from those of the other strains tested in that it was not inhibited by cloxacillin, clavulanic acid, or sulbactam. In contrast, all of the other strains produced classical type I activities as characterized by pI and inhibition to a much greater extent by cloxacillin than by clavulanic acid and sulbactam (14).

Hydrolysis of cephaloridine, cefepime, ceftazidime, and cefotaxime by the β-lactamases in these strains was examined. Cephaloridine was hydrolyzed at significant rates; 38.3 and 145 nmol/min per mg of protein for cefepime-susceptible strains A26401 and A21481, respectively, compared with 285 to 4,412 nmol/min per mg of protein for cefepime-resistant strains. No significant hydrolysis of cefepime, ceftazidime, or cefotaxime was detected over the 4 min of monitoring at enzyme concentrations capable of hydrolyzing up to 0.1 mmol of cephaloridine per min.

Cefepime susceptibility of high β-lactamase producers of members of the family *Enterobacteriaceae*. Cefepime remained active against strains of *E. cloacae* and *C. freundii* which constitutively produced high levels of β-lactamase (Table 3). Cefotaxime and ceftazidime resistances in *E. cloacae* and *C. freundii* were associated with 150- to 750-fold increases in β-lactamase levels. The cefepime MICs for

TABLE 3. MICs and β-lactamase levels in *Enterobacteriaceae*

Organism and strain	MIC (μg/ml)			β-Lactamase sp act (mU/mg)
	Cefepime	Ceftazidime	Cefotaxime	
E. cloacae				
A20476	0.06	0.25	0.25	18.4
A20936	0.03	0.13	0.13	30
A20495	1	128	64	2,836
A26289	4	>128	>128	14,063
C. freundii				
A22160	0.015	0.25	0.06	21.3
A22141	0.13	1–2	0.5	15.4
A21312	0.015	0.13	0.13	5.7
A26183	1–2	>128	64	12,048

TABLE 4. Effect of β-lactamase induction on cephalosporin MICs

Cephalosporin	MIC (μg/ml) for:			
	E. cloacae A15156		*P. aeruginosa* A9843	
	Noninduced	Induced[a]	Noninduced	Induced[a]
Cefepime	0.13	0.13	4	16
Ceftazidime	1	8	2	32
Cefotaxime	1	16	16	>128

[a] After 2 h of growth at 1/8 of the MIC of imipenem.

these high-level-β-lactamase-producing strains were higher than those for low-level-β-lactamase-producing strains, but in each case, the cefepime MIC was 4 μg/ml or lower.

Effect of β-lactamase induction on cephalosporin MICs. The MICs of *E. cloacae* A15156 and *P. aeruginosa* A9843 containing inducible β-lactamase were determined pre- and postinduction (Table 4). In both strains, the induced level of β-lactamase was 10-fold higher than in the noninduced cultures. The higher β-lactamase levels in both strains were accompanied by 8- to 16-fold increases in the ceftazidime and cefotaxime MICs. Cefepime MICs were unaffected by induction of *E. cloacae* A15156 and increased fourfold for *P. aeruginosa* A9843.

DISCUSSION

Although the MICs of cefepime for ceftazidime-cefotaxime-resistant strains are higher than those for susceptible strains, they are increased proportionately less such that cross-resistance (MIC, >16 μg/ml) between cefepime and the broad-spectrum cephalosporins ceftazidime, cefotaxime, cefoperazone, and ceftriaxone is low. Among the ceftazidime-cefotaxime-resistant clinical isolates, cross-resistance was observed for cefepime only with *P. aeruginosa* and only in 6 of the 32 ceftazidime-cefotaxime-resistant strains tested. This is consistent with the rare cross-resistance previously observed between cefepime and cefotaxime-ceftazidime-resistant mutants derived in vitro (4).

In this study, we were unable to associate prior antibiotic exposure with resistance to cefepime, since only limited clinical data on the six cefepime-resistant strains could be obtained. Only in the case of strain A26404 (a wound isolate) was prior antimicrobial therapy (ceftazidime) identified.

Although high β-lactamase levels may be achieved via induction or stable derepression, clinical isolates resistant to multiple β-lactam antibiotics are predominantly derepressed mutants (2, 3, 18). We previously reported that among cefepime-susceptible strains of *E. coli*, *E. aerogenes*, and *E. cloacae*, higher β-lactamase levels were observed in ceftazidime-resistant strains than in ceftazidime-susceptible strains (12). In this study, we found that to obtain cefepime resistance in *P. aeruginosa* even higher β-lactamase levels were required than for ceftazidime resistance alone. The high β-lactamase production was stably derepressed in cefepime-resistant clinical isolates of *P. aeruginosa*. *P. aeruginosa* strains (A25058, A26316, and A26403) with cefepime MICs close to the breakpoint (16 to 32 μg/ml) contained moderately high levels of β-lactamase, which were higher than those observed with strains consistently exhibiting cefepime MICs of 16 μg/ml or lower and lower than those of strains consistently exhibiting cefepime MICs of 32 μg/ml or greater. Thus, the direct relationship of β-lactamase levels of cefepime susceptibility has now been observed with clinical isolates as well as with mutants derived in vitro (4). The results of the induction experiments shown in Table 4 also confirm the greater impact of increased β-lactamase levels on the MICs of ceftazidime and cefotaxime than on the MIC of cefepime, since the association of elevated β-lactamase levels with the magnitude of the MIC increase is common to both induced cells and derepressed mutants. No outer membrane protein profile changes were seen in derepressed cells (data not shown). It is unlikely that induced cells and derepressed mutants would have more than one resistance mechanism in common.

As with cefepime-susceptible strains (12), cefepime has a low affinity for the β-lactamases of cefepime-resistant strains and is highly resistant to enzymatic hydrolysis. The lower affinity of cefepime for β-lactamases combined with better penetration through the outer membrane may explain the retention of activity of cefepime against ceftazidime-resistant strains. The rate of penetration of cefepime through the OmpF porin of *E. coli* is at least sixfold higher than that of ceftazidime (H. Nikaido, personal communication). Even though cefepime is slightly more susceptible to hydrolysis than is ceftazidime at a high (100 μM) substrate concentration (5, 12, 17), the hydrolysis velocities at 10 and 0.1 μM with high concentrations of enzymes from *E. cloacae* and *C. freundii* were lower for cefepime than ceftazidime. The greater reduction in hydrolysis velocity at lower substrate concentrations for cefepime relative to that observed for ceftazidime (5) could result from the difference between the initial binding affinities of these two cephalosporins. The overall result is that the net accessibility to the target penicillin-binding proteins may be higher for cefepime.

Although zwitterionic compounds such as cefepime penetrate through porin channels much more rapidly than do compounds carrying a net negative charge, the cefepime resistance encountered only in *P. aeruginosa* isolates may be due to the overall poorer penetration of β-lactams in this species (10). The penetration of β-lactams, in general, is about 100- to 500-fold lower in *P. aeruginosa* than in *E. coli*. Overproduction of β-lactamase coupled with more limited penetration of the compound may result in resistance-level MICs for *P. aeruginosa* strains not seen for other gram-negative bacteria. As shown in this study, cefepime MICs for *Enterobacteriaceae* increased with β-lactamase overproduction but the MICs remained in the susceptible range, presumably because permeability is less restricted.

In conclusion, cefepime resistance was observed only in a subset of strains of *P. aeruginosa* which are resistant to ceftazidime and cefotaxime. Cefepime-resistant *P. aeruginosa* isolates constitutively produce high amounts of β-lactamase at levels even higher than those observed in ceftazidime-resistant–cefepime-susceptible strains. All other gram-negative isolates which have gained resistance to ceftazidime and/or cefotaxime are susceptible to cefepime. These include strains producing CTX-1 (TEM-3), one of the newer plasmid-encoded β-lactamases (D. C. Sirot, C. Chanal, J. Sirot, R. Labia, and R. Cluzel, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 515, 1987). It will be of interest to see the activity of cefepime against strains producing other new enzymes like SHV-2 and CAZ-1. The low cross-resistance with broad-spectrum cephalosporins and the lower potential to select resistance (4) clearly demarcate cefepime from previous broad-spectrum compounds and suggest that resistance during clinical use is much less probable.

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